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CD3-CD28 Costimulation as a Means to Avoiding T Cell Preactivation in Bispecific Monoclonal Antibody-based Treatment of Ovarian Carcinoma[1]

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ABSTRACT

One of the major limitations to the immunotherapy of ovarian carcinoma based on the use of anti-CD3/antitumor bispecific monoclonal antibodies (bi-mAb) is the need for preactivation of effector cells *ex vivo*, because cross-linking of the T cell receptor-CD3 complex *per se* may lead to T-cell unresponsiveness or even apoptosis. The bi-mAb OC/TR, which recognizes the folate-binding protein (FBP) overexpressed in 90% of ovarian carcinomas and the CD3 molecule on T cells, has demonstrated efficacy in a clinical setting. Here we investigated the possibility of delivering accessory signals to OC/TR-retargeted peripheral blood mononuclear cells (PBMCs) via an anti-CD28 mAb or an anti-FBP/anti-CD28 bi-mAb.

Coculture of resting PBMCs from healthy donors with OC/TR, anti-FBP/anti-CD28 bi-mAb, and FBP$^+$ tumor cell lines resulted in a highly activated phenotype of effector cells and in a dramatic *in vitro* growth inhibition of the target cells without an increase in OC/TR-redirected lysis. Whereas both the CD4 and CD8 T cell subsets were involved in the growth inhibition, only the CD8 subpopulation accounted for the cytotoxic activity. The *in vitro* tumor growth inhibition was mediated mainly by soluble factors, which were active on both FBP$^+$ and FBP$^-$ ("bystander effect") cell lines. Activation and antitumor activity were also observed, albeit to a lesser extent, using OC/TR and monospecific bivalent anti-CD28 mAb. *In vitro* analysis demonstrated that cross-linking between tumor and effector cells for at least 24 h was needed to achieve T-cell activation and development of antitumor activities. Thus, *ex vivo* CD3-CD28 costimulation on resting PBMCs might be of therapeutic utility for local treatment of minimal residual disease.

INTRODUCTION

bi-mAbs[4] directed against the TCR/CD3 complex on T lymphocytes and antigenic structures on tumor cells can target polyclonal immune effectors toward tumor cells in an MHC-unrestricted manner, irrespective of their natural specificity (reviewed in Ref. 1). Yet engagement of the TCR/CD3 complex *per se* is not sufficient to induce the complete array of T-cell effector functions (*i.e.*, cytotoxicity, cytokine secretion, and proliferation), and even when retargeted by bi-mAb against tumor cells, resting T lymphocytes have little, if any, lytic activity (2–4). Moreover, cross-linking of the TCR/CD3 complex alone may lead to a state of T-cell unresponsiveness (anergy) or even to cell death by apoptosis (5).

Thus, adoptive immunotherapy based on the use of anti-CD3/anti-TAA bi-mAb requires the *ex vivo* activation of T cells for effective antitumor reactions. T-cell activation can be achieved by incubation with anti-CD3 mAb in conjunction with mitogenic lectins or cytokines, and the resulting effector cells have been used successfully in several *in vitro* as well as *in vivo* models for the lysis, growth inhibition, and eradication of many different tumors (3, 6–12).

In bi-mAb-based therapy for patients with solid tumors, treatment was administered locoregionally after *ex vivo* activation of autologous T cells with IL-2 alone or plus phytohemagglutinin and subsequent coating with bi-mAb (13, 14). Despite favorable results achieved in both cases, the generation of effector cells *ex vivo* must be approached with caution since various technical and theoretical considerations can reduce their effectiveness. In fact, *in vitro* expansion and activation of T cells is a time-consuming, expensive procedure that requires specialized personnel and carries the risk of cell culture contamination. Moreover, it is likely to alter the trafficking properties of lymphocytes, thereby reducing the antitumor efficacy of the treatment.

On the basis of the two-signal model for T cell activation, anti-CD3 mAb-induced anergy can be circumvented by delivering costimulatory signals to lymphocytes. The costimuli are physiologically provided by interactions between non-antigen-specific accessory receptors on the T-cell surface and their ligands expressed by antigen-presenting cells. Among several molecules known to support T-cell activation, the CD28 homodimer appears to play a major role (reviewed in Refs. 15 and 16). Whereas CD28 stimulation (by its counter-receptors B7-1 and B7-2 as well as by anti-CD28 mAb) does not by itself induce any response in T cells, simultaneous ligation of CD28 and TCR/CD3 dramatically augments the production of a variety of cytokines and results in rapid T-cell proliferation and differentiation into effector cells.

The combination of bi-mAb directed against CD3 or CD28 on the one hand and a TAA on the other hand has been suggested by Jung *et al.* (17, 18) to achieve a target cell-induced T-cell activation resulting in tumor cell destruction. The effectiveness of this model *in vitro* and in mice carrying established xenografted Hodgkin's tumor is well established (17–25). However, no clinical trials using a combination of CD3 and CD28-specific bi-mAb have yet been initiated.

We have developed a mAb, MOv18, that recognizes FBP that is overexpressed in about 90% of ovarian carcinomas (26, 27). The MOv18-producing hybridoma was used to obtain the bi-mAb OC/TR that shows specificities toward both the FBP and CD3 molecules (anti-FBP/anti-CD3; Refs. 28 and 29). Retargeting of *in vitro*-activated peripheral blood T lymphocytes from healthy donors and from patients with ovarian carcinoma using OC/TR resulted in specific lysis of ovarian carcinoma cells *in vitro* (3) and in tumor regression in a xenogeneic model (30, 31). Moreover, tumor regression occurred in 27% of patients with advanced ovarian cancer after i.p. administration of *in vitro* activated and expanded lymphocytes targeted with OC/TR (14).

In an attempt to shorten and possibly to avoid the *in vitro* preactivation of T lymphocytes, the role of accessory signals delivered to OC/TR-retargeted resting T cells via an anti-CD28 mAb or an anti-FBP/anti-CD28 bi-mAb was evaluated. Here we show that the simultaneous presence of the anti-CD3- and anti-CD28-containing bi-mAb

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[4] The abbreviations used are: bi-mAb, bispecific monoclonal antibody; TAA, tumor-associated antigen; IL, interleukin; TCR, T-cell receptor; FBP, folate-binding protein; PBMC, peripheral blood mononuclear cell; PE, phycoerythrin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; TNF, tumor necrosis factor; CTLL, cytotoxic T cell line.

dramatically increases the antitumor activity of freshly isolated PBMCs by a cytokine-mediated mechanism.

MATERIALS AND METHODS

Monoclonal Antibodies. The bi-mAb OC/TR (IgG1/IgG1), specific for the ovarian carcinoma-associated antigen FBP and the CD3 molecule, was produced by fusion of hybridoma Mov18 with spleen cells from a BALB/c mouse immunized with a human T-cell clone (28). The $F(ab')_2$ fragments of the bi-mAb OC/TR were provided in purified form by Centocor Europe (Leiden, The Netherlands). The anti-CD28 mAb 9.3 (IgG2a) and its $F(ab')_2$ fragments were produced as described previously (32).

The bispecific $F(ab')_2$-hybrid mAb with FBP and CD28 specificities (hereafter named FBP/CD28 bi-mAb) was prepared by selective reduction and reoxidation of hinge region disulfide bridges as described previously (32). Briefly, $F(ab')_2$ fragments of the MOv18 and 9.3 antibodies were reduced and modified using a 2-nitro-5-thiobenzoate-anion/5,5-dithio-bis-(2-nitrobenzoic acid) mixture. The resulting 2-nitro-5-thiobenzoate-anion-modified Fab fragments could be hybridized after re-reduction of one of the modified fragments. Using this method, 50–60% of the Fab material could be hybridized to form bispecific $F(ab')_2$ fragments.

Unless otherwise stated, each mAb was used at a final concentration of 1 μg/ml.

Cell Lines. Two human FBP^+ ovarian carcinoma cell lines were used: IGROV1, a gift from Dr. J. Bernard (Institute Gustave Roussy, Villejuife, France), and OVCAR3, provided by the American Type Culture Collection (Rockville, MD). The human FBP^- epidermoid carcinoma cell line A431 was from American Type Culture Collection. The cell lines, as well as PBMCs (see below), were grown in RPMI 1640 supplemented with 10% FCS, 2 mM glutamine, and 100 μg/ml streptomycin (culture medium) in a humidified atmosphere of 5% CO_2 at 37°C. Cells were routinely tested for *Mycoplasma* contamination and were consistently negative.

The reactivity of mAb used in this study on target and effector cells was evaluated by flow cytometry. OC/TR strongly binds the FBP^+ ovarian carcinoma cell lines through its MOv18 component but not the unrelated FBP^- line A431 and binds freshly isolated PBMCs through its anti-CD3 component. The reactivity of the bi-mAb on tumor cells and on PBMCs is comparable to that of the parental mAb, despite its monovalency. The FBP/CD28 bi-mAb shows the same tumor cell binding pattern as OC/TR and stains PBMCs as 9.3 does.

To exclude the possibility that target cells by themselves provide costimulatory signals to T cells, tumor cells were tested for surface expression of the CD28 ligand B7-1 and were consistently negative.

T-Cell Separation and Culture. PBMC were isolated from buffy coats of healthy donors by Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) density gradient centrifugation. $CD4^+$ and $CD8^+$ T-cell subsets were purified from PBMCs using the Mini-MACS magnetic cell sorting system (Miltenyi Biotec GmbH, Sunnyvale, CA). After monocyte depletion by overnight plastic adherence at 37°C, lymphocytes were incubated with anti-CD4-coated microbeads and subsequently separated according to the manufacturer's instructions. The purity of the two subsets was assessed by flow cytometry. $CD8^+$ cells were enriched 2.5–3-fold in the unbound fraction, and no contamination of $CD4^+$ cells was detected. More than 99% of the cells eluted from beads were $CD4^+$, and no cells were stained for CD8.

PBMCs were cultured at a concentration of 10^6 cells/ml in culture medium in the presence of FBP^+ target cells (E:T, 20:1) and various mAb combinations (stimulation mixture). At various time intervals, PBMCs were collected and counted, and their phenotypes were characterized by flow cytometry; on day 3, PBMCs were tested for their cytotoxic and tumor growth inhibition ability.

Flow Cytometry. The following antibodies were used: anti-CD3 (Leu-4)-FITC or -PE, anti-CD4 (Leu-3a)-PE, anti-CD8 (Leu-2b)-FITC, anti-CD14 (Leu-M3)-FITC, anti-CD16 (Leu-11c)-PE, anti-CD25 (anti-IL-2R)-FITC or -PE, anti-CD28 (Leu-28)-PE, anti-CD69 (Leu-23)-PE, and anti-CD80 (anti-BB1/B7)-PE were all from Becton Dickinson (San Jose, CA). For the detection of mouse antibodies, a FITC-conjugated goat antimouse IgG and IgM were used (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD). Stained cells were analyzed on a FACScan (Becton Dickinson) using Lysys II software. Five thousand cells were acquired for each sample; cells were gated for lymphocytes using forward and side scatter.

Cytotoxicity Assay. Cytotoxicity was determined in a standard 4-h ^{51}Cr release assay. Target cells were incubated for 1 h at 37°C with 100 μCi $Na_2{}^{51}CrO_4/10^6$cells (ICN Biomedicals, Milan, Italy). After washing, cells (5000/well) were seeded in triplicate in U-shaped 96-well plates at different E:T ratios, ranging from 100:1 to 5:1 and various mAb combinations in a final volume of 0.2 ml/well. After 4 h of incubation, 0.1 ml/well of supernatants was withdrawn, and radioactivity was counted in a beta counter. Spontaneous release of ^{51}Cr, determined in cells incubated with medium alone, was always <20%. Maximum ^{51}Cr release was obtained by lysing the cells in 1% NP40. The SE of triplicates never exceeded 5–10%. The percentage of specific lysis was calculated as:

$$100 \times \frac{\text{Experimental release cpm} - \text{spontaneous release cpm}}{\text{Maximum release cpm} - \text{spontaneous release cpm}}$$

Tumor Growth Inhibition Assay. Tumor growth inhibition was evaluated by a colorimetric assay with MTT (Sigma Chemical Co., St. Louis, MO) as described (33). Target cells were seeded (7000/well in 0.1 ml for IGROV1 and OVCAR3, 1000/well in 0.1 ml for A431) in triplicate in flat-bottomed 96-well plates, with different numbers of effector cells and various mAb combinations in 0.1 ml of medium. After a 7-day incubation at 37°C, each well was washed twice to remove nonadherent cells (effector cells or dead target cells), and 0.1 ml/well of fresh medium containing 0.5 mg/ml MTT was added. Cells were incubated for at least 4 h at 37°C, and 0.1 ml of 2-propanol was added to each well, mixed thoroughly; absorbance at 570 nm was determined in a micro-ELISA reader (Titertek Multiskan; Flow Laboratories). The percentage of growth inhibition was calculated as:

$$100 - 100 \times \frac{\text{A sample} - \text{A medium}}{\text{A control} - \text{A medium}}$$

where target cells grown in medium alone represent the control. The SE of triplicates never exceeded 5–10%.

To confirm the cooperative activity of anti-CD28 mAb or FBP/CD28 bi-mAb and OC/TR on adherent cells, target cells were seeded 24 h before the addition of PBMCs and antibodies so as not to prevent their adhesion to the plastic support. Growth inhibition was evaluated as above.

For the evaluation of bystander cell growth inhibition, OVCAR3 cells were fixed with 1% paraformaldehyde in PBS for 30 min on ice. Cells were extensively washed with medium and seeded at 7000 cells/well. Titrated amounts of resting PBMCs were added, together with 1000 cells/well of A431 and various mAb combinations. Plates were incubated for 7 days at 37°C; A431 cell growth was evaluated as above.

Proliferation Assay. PBMCs were seeded in 48-well plates at 1×10^6/well in 1 ml/well of culture medium in the presence of FBP^+ target cells (E:T ratio, 20:1) and various mAb combinations. On days 3, 5, 7, and 10, PBMCs were collected, washed, and counted by trypan blue exclusion. The SE of triplicates never exceeded 5–10%.

Determination of Cytokine Levels. Supernatants from stimulation mixtures (see "T-Cell Separation and Culture") were collected, sterile-filtered (pore size, 0.2 μm), and tested immediately or stored at −20°C. TNF-α and IFN-γ levels were determined using ELISA kits (R&D Systems, Minneapolis, MN and Genzyme Corp., Cambridge, MA, respectively), following the manufacturer's instructions. The lower limit of detection was 16 pg/ml in both cases.

IL-2 was measured with an IL-2-dependent CTLL proliferation assay. Briefly, 10^4 CTLL cells/0.1 ml/well were incubated with 0.1 ml of supernatants from the stimulation mixtures at 37°C in 96-w microtiter plates. One day later, 1 μCi/w of [^{3}H]thymidine (Amersham, Aylesbury, United Kingdom) was added, and after 18 h of incubation, the cells were harvested, and radioactivity was counted in a beta counter. The levels of IL-2 found in supernatants were extrapolated from a titration curve obtained incubating CTLL cells with human recombinant IL-2 (EuroCetus, Amsterdam, the Netherlands) ranging from 16.6 to 0.032 ng/ml.

The ability of supernatants to inhibit the growth of tumor cells was tested in an MTT assay as described above, incubating 0.1 ml/well of each supernatant with tumor cells.

RESULTS

Retargeting of Freshly Isolated PBMCs. At different E:T ratios, PBMCs retargeted by the bi-mAb OC/TR display a limited but detectable lytic activity toward FBP^+ but not FBP^- target cells. The addition of anti-CD28 antibody, either cross-linked to the target cells (by FBP/CD28 bi-mAb) or in soluble form (anti-CD28 mAb), did not induce any significant increase in cytotoxicity. Similar results were obtained with PBMCs from 13 different donors (data not shown) using IGROV1 or OVCAR3 FBP^+ cells.

Cytotoxicity assays with sorted lymphocyte subpopulations demonstrated that all of the OC/TR-targetable cytotoxicity was present in the $CD8^+$-enriched population. Although the cells enriched for CD8 contained also some natural killer cells, the cytotoxicity was mediated by T cells because no lysis was detectable in the absence of OC/TR, even at high E:T ratios. Again, the simultaneous presence of the anti-CD28 mAb or FBP/CD28 bi-mAb did not increase OC/TR-mediated tumor lysis (data not shown).

PBMCs were also tested for their ability to inhibit the growth of tumor cells *in vitro*. OC/TR-retargeted PBMCs were able to inhibit the growth of FBP^+ target cells, and this activity was significantly increased by the addition of the anti-CD28 mAb, an effect particularly evident at low E:T ratios (around 2:1; Fig. 1*A*). In 16 different donors, the anti-CD28 mAb and its $F(ab')_2$ increased the antitumor activity of targeted PBMCs by an average of 39.7% ($P < 0.05$ by Student's t paired test; Fig. 1*B*), and that was further increased by using the FBP/CD28 bi-mAb, especially at E:T ratios below 1:1 (Fig. 1*C*).

The anti-CD28 mAb and the FBP/CD28 bi-mAb increased the growth inhibition of FBP^+ target cells by OC/TR-retargeted PBMCs over a wide range of concentrations, reaching a plateau at about 20 ng/ml (data not shown). The tumor growth inhibition obtained with the anti-CD28 mAb or the FBP/CD28 bimAb in the absence of OC/TR was comparable to that achieved with PBMCs alone.

MTT assays with sorted lymphocyte subpopulations showed that the increase in tumor growth inhibition obtained upon the addition of anti-CD28 mAb or FBP/CD28 bi-mAb to OC/TR-retargeted PBMCs occurred with both the $CD4^+$-purified and the $CD8^+$-enriched populations (Table 1), although a difference between the two subsets was observed in the activity of OC/TR-redirected T cells in the absence of costimuli. In fact, in the case of the $CD8^+$-enriched population, the tumor growth inhibition was much higher than for the $CD4^+$ subset at any E:T ratio tested. Identical results were obtained using a sorted $CD8^-$ population (100% $CD8^+$) instead of the $CD8^+$-enriched subset (data not shown).

No tumor growth inhibition was observed on FBP^- target cells, even in the presence of both bi-mAbs together and at the highest E:T ratio tested; growth inhibition was never over background levels, *i.e.*, 10% (Table 2).

The anti-CD28 mAb and the FBP/CD28 bi-mAb potentiated the ability of OC/TR-retargeted PBMCs to block the growth also of adherent FBP^+ target cells, excluding that the effect was only due to the prevention of target cell adhesion (data not shown).

Phenotype Characterization. T-cell activation in the presence of different mAb combinations and FBP^+ target cells was monitored at different time points. Fig. 2, *A* and *B*, shows that by day 3, the PBMCs were activated, as judged by CD69 and CD25 expression. CD69 increases were seen long after 6 h of incubation, whereas CD25 up-regulation became detectable after 24 h of incubation, reaching maximal levels by day 3. CD69 expression was nearly independent of CD28 costimulation; by contrast, CD25 expression increased markedly as a result of CD28 costimulation. The rise in CD25 expression induced by OC/TR alone or plus anti-CD28 mAb or FBP/CD28 bi-mAb was transient and declined starting from day 4 of incubation.

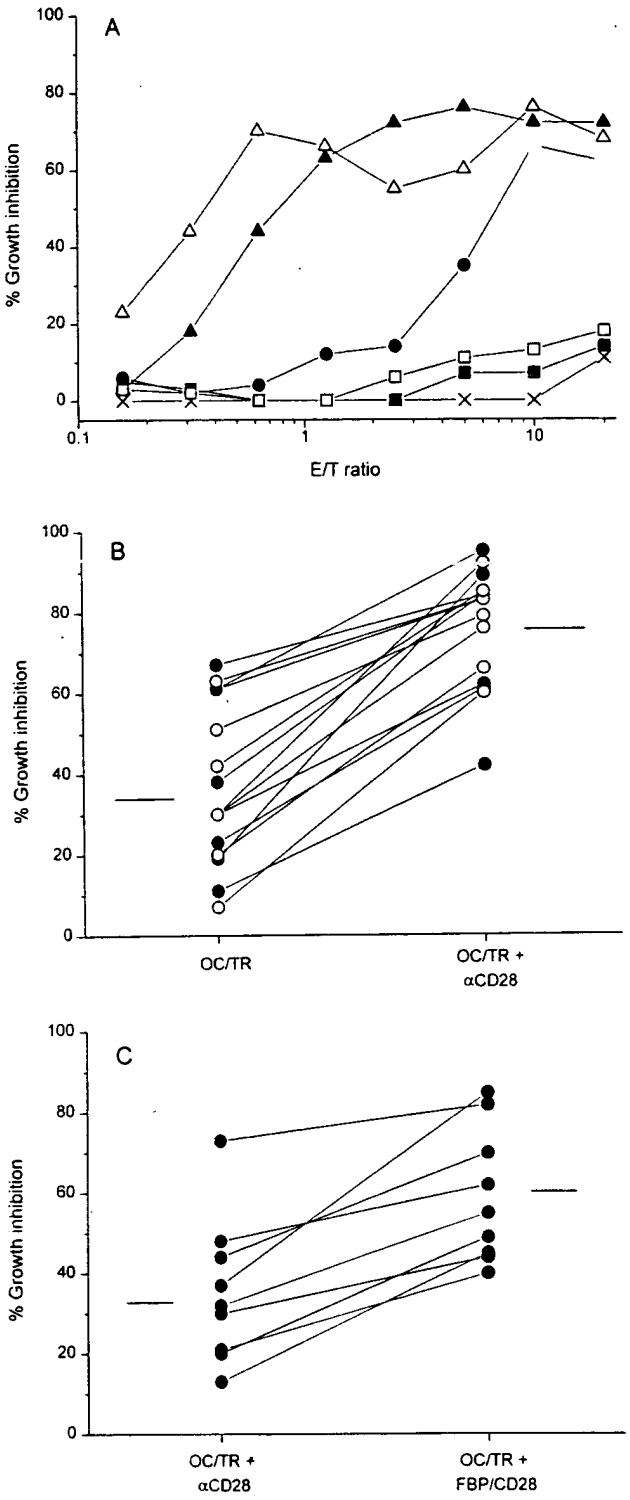

Fig. 1. Potentiation of OC/TR-mediated tumor growth inhibition by resting PBMCs after the addition of anti-CD28 mAb or FBP/CD28 bi-mAb. *A*, inhibition of FBP^- OVCAR3 growth by resting PBMCs. PBMCs were seeded at the indicated E:T (*E/T*) ratios with target cells in the presence of: medium alone (×), OC/TR (●), OC/TR plus anti-CD28 mAb (▲), OC/TR plus FBP/CD28 bi-mAb (△), anti-CD28 mAb (■), and FBP/CD28 bi-mAb (□). All of the mAbs were in $F(ab')_2$ form. Data are from one representative experiment of 16 performed. *B*, growth inhibition activity by CD28 cross-linking via anti-CD28 mAb in 16 different donors. The assay was performed at an E:T ratio of 2.5:1 using IGROV1 or OVCAR3 cells as FBP^+ targets and anti-CD28 mAb in intact ( ) or in $F(ab')_2$ form (○). *Horizontal lines* indicate average growth inhibition. *C*, comparison between anti-CD28 mAb and FBP/CD28 bi-mAb costimulatory activity. Data are from nine different donors at an E:T ratio of 0.6:1. Target cells were OVCAR3. *Horizontal lines* indicate average growth inhibition.

This pattern of activation was present in both $CD4^+$ and $CD8^+$ cells; however, in $CD8^+$ cells, the CD28 costimulation did not further increase CD25 expression.

The anti-CD28 mAb, but not the FBP/CD28 bi-mAb, down-modulated CD28 from the surface of T cells, beginning 1 h after engagement and persisting long after (Fig. 2*C*). The CD28 molecule reappeared totally 7 days after stimulation.

No changes in the percentage of $CD3^+$ cells and no clearcut stimulation of $CD4^+$ or $CD8^+$ populations were detected in any of the mAb combinations tested. On day 3 after stimulation, we were unable to find any definite, preferential, or reproducible expansion of either subpopulation.

Proliferative Effect of mAb Combinations on PBMCs. After 3 days of culture with FBP^+ target cells and mAb, no increase in the number of T cells was detected, independent of the mAb combination used for stimulation. The analysis of proliferation over the next 7 days in the absence of exogenous cytokines revealed substantial growth only in PBMCs cultured with OC/TR and the FBP/CD28 bi-mAb; cell recovery after 10 days of culture ranged from 156 to 228% of seeded cells. In all other groups of lymphocytes, proliferation was decreased (50–90% recovered; Fig. 3).

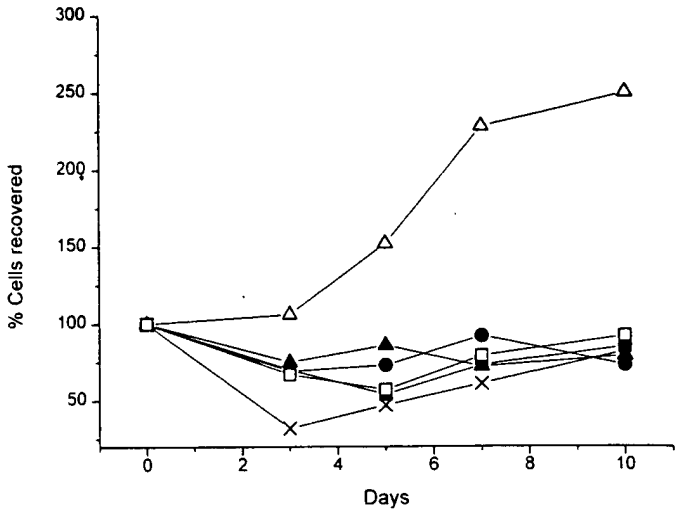

Fig. 3. PBMC proliferation following stimulation with different mAb combinations in the presence of FBP^+ target cells. On day 0, resting PBMCs were seeded with OVCAR3 cells (E:T ratio, 20:1) and the following mAb: none (×); OC/TR ( ); OC/TR plus anti-CD28 mAb (▲); OC/TR plus FBP/CD28 bi-mAb (△); anti-CD28 mAb (■); and FBP/CD28 bi-mAb (□). All of the mAbs were in $F(ab')_2$ form. On the indicated days, lymphocytes were collected, washed, and counted by trypan blue exclusion. Data are from a representative experiment of three performed with superimposable results.

Table 1 *Growth inhibition of FBP^+ target cells in vitro by sorted resting lymphocytes*[a]

mAb[b]	% OVCAR3 cell growth inhibition by: Total PBMC	$CD8^+$	$CD4^+$
None	15	9	6
OC/TR	40	34	16
OC/TR + αCD28	75	76	72
OC/TR + FBP/CD28	86	87	85
αCD28 mAb	5	0	0
FBP/CD28	14	10	3

[a] Evaluated at an E:T of 1.25:1. $CD8^+$ and $CD4^+$ subsets were 63 and 100% pure, respectively. Data were from one of six experiments performed.
[b] All mAbs were in $F(ab')_2$ form.

Table 2 *Growth inhibition of A431 bystander cells by resting PBMCs*[a]

mAb[b]	No OVCAR3	Plus OVCAR3
None	1.2 ± 1.2	2.5 ± 1.0
OC/TR	2.5 ± 2.5	7.0 ± 4.3
OC/TR + αCD28	2.0 ± 2.0	14.7 ± 3.1
OC/TR + FBP/CD28	1.5 ± 1.5	26.7 ± 5.1
αCD28	2.0 ± 2.0	3.0 ± 1.8
FBP/CD28	2.0 ± 2.0	3.2 ± 2.1

[a] Resting PBMCs were incubated for 7 days at an initial E:T ratio of 17.5:1 and 2.5:1 with respect to live FBP^- A431 and fixed FBP^+ OVCAR3 cells, respectively. Data are expressed as the mean of the percentage of growth inhibition ± SE of four independent determinations.
[b] All mAbs were in $F(ab')_2$ form.

Lytic and Tumor Growth Inhibition Activity after 3 Days of Stimulation. PBMCs cultured in the presence of OC/TR (alone or plus anti-CD28 mAb or FBP/CD28 bi-mAb) and FBP^+ cells for 3 days were still able to lyse FBP^+ target cells (Fig. 4*A*) and to inhibit their growth (Fig. 4*B*). Thus, residual bi-mAb OC/TR was present on the cell membrane and fully retained its retargeting activity. The cytotoxicity was significantly higher as compared with freshly isolated PBMCs, whereas at a relatively high E:T ratio (1:1), their ability to inhibit the tumor growth was only slightly increased. Despite the higher degree of activation (Fig. 2*B*), the lytic activity of PBMCs incubated with target cells in the presence of OC/TR plus anti-CD28

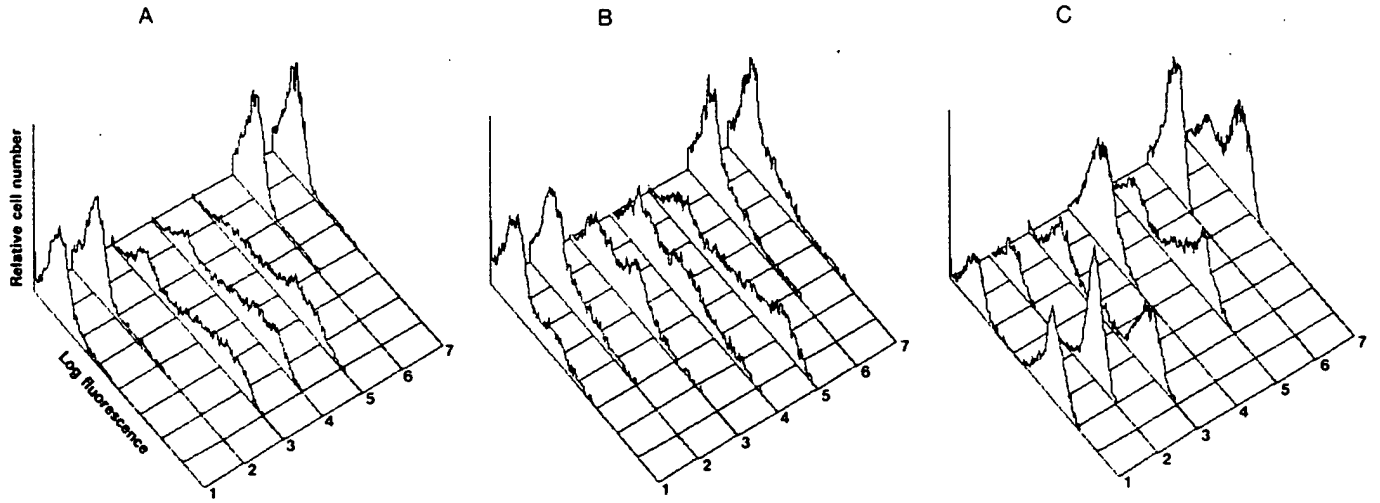

Fig. 2. Phenotypic analysis of PBMCs cultured in the presence of FBP^+ target cells and different mAb combinations. Freshly isolated PBMCs (*profile 1*) were cocultured with IGROV1 cells (E:T ratio, 20:1) and the following mAb combinations: none (*profile 2*); OC/TR (*profile 3*); OC/TR plus anti-CD28 mAb (*profile 4*); OC/TR plus FBP/CD28 bi-mAb (*profile 5*); anti-CD28 mAb (*profile 6*); and FBP/CD28 bi-mAb (*profile 7*). Antibodies were used in their $F(ab')_2$ form. On day 3, lymphocytes were collected, washed, and analyzed by flow cytometry for the expression of: *A*, CD69; *B*, CD25; and *C*, CD28. The experiment was repeated 10 times with similar results.

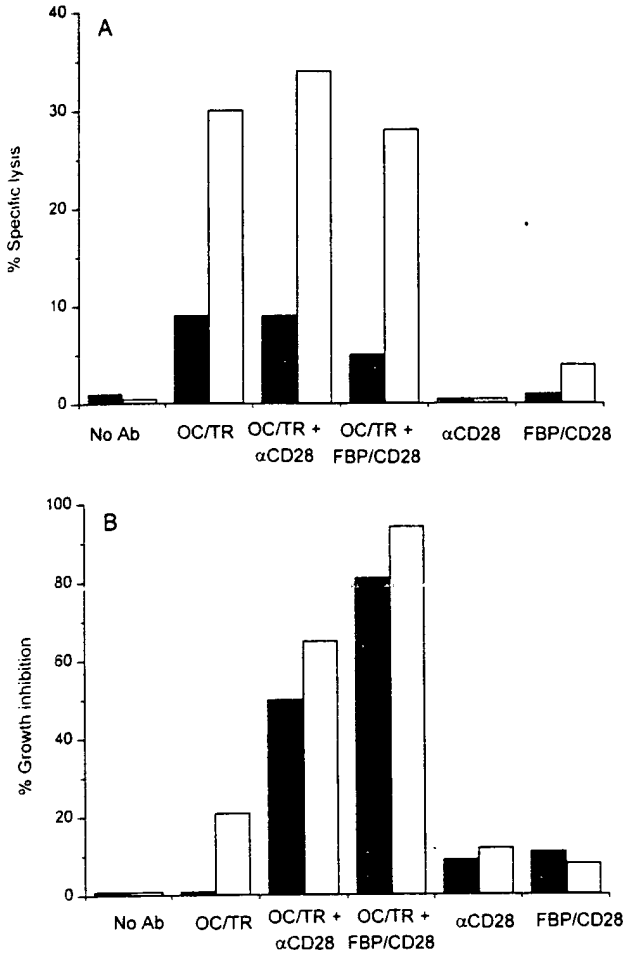

Fig. 4. Cytotoxicity and tumor growth inhibition activity of freshly isolated *versus* stimulated PBMCs. Freshly isolated PBMCs were assayed immediately after isolation (■) or after coculture for 3 days with OVCAR3 cells (E:T ratio, 20:1) and various mAb combinations (□). *A*, cytotoxicity in a 4-h ^{51}Cr release assay. *B*, ability to block the growth of tumor cells in a 7-day MTT assay. A representative experiment on OVCAR3 target cells is shown at an E:T ratio of 6:1 (*A*) and 1:1 (*B*).

(either anti-CD28 mAb or FBP/CD28 bi-mAb) was not higher than that of OC/TR-activated T cells, whereas tumor growth inhibition activity was considerably higher than that obtained with OC/TR alone, consistent with the data obtained with resting PBMCs. PBMCs incubated in the absence of antibodies or with anti-CD28 alone (anti-CD28 mAb or FBP/CD28 bi-mAb) showed no change in their lytic or inhibitory activities. The addition of bi-mAb OC/TR to PBMCs activated previously with OC/TR did not significantly increase the cytotoxicity or *in vitro* tumor growth inhibition (data not shown). No lysis or growth inhibition of FBP^- A431 cells was detected at any E:T ratio tested by stimulated PBMC (data not shown).

Bystander Effect. To assess whether the growth of bystander cells was inhibited, supporting a cytokine-mediated mechanism, FBP^- A431 cells were seeded with titrated numbers of PBMCs and various mAb combinations, in the presence or absence of paraformaldehyde-fixed FBP^+ OVCAR3 cells. After 7 days, the inhibition of A431 cell growth was evaluated. In the absence of OVCAR3 cells, no growth inhibition of A431 cells was observed, since no cross-linking of OC/TR occurred; on the other hand, in the presence of fixed FBP^+ OVCAR3 cells, A431 cell growth was significantly inhibited, provided that OC/TR was added to the culture. When the anti-CD28 mAb was also present, a slight increase in growth inhibition was observed, but only the addition of FBP/CD28 bi-mAb elicited maximum tumor cell growth inhibition (Table 2).

Supernatant Analysis. To determine whether the bystander effect was due to soluble factors released by PBMCs following cross-linking of their TCR/CD3 complexes, supernatants were collected on day 3 from the stimulation mixtures and analyzed in an MTT assay. Both FBP^+ and FBP^- target cells were significantly inhibited by a 2-fold dilution of the supernatants recovered from OC/TR-activated PBMC cultures; moreover, the addition of an anti-CD28, in particular of FBP/CD28 bi-mAb, to the stimulation mixtures resulted in a higher degree of tumor growth inhibition (Table 3) by the supernatants, which correlated well with the extent of activation achieved by the corresponding PBMCs.

To identify some of the factors responsible for blocking tumor growth, supernatants were assayed for the presence of IFN-γ and TNF-α. High titers of both cytokines were detected, especially in those collected from OC/TR plus FBP/CD28 bi-mAb-stimulation mixtures (Table 3).

IL-2 was detected only in supernatants derived from OC/TR plus FBP/CD28 bi-mAb stimulation mixtures (three different donors were tested, with a mean of 1.22 ng/ml; range, 0.514–1.77 ng/ml).

DISCUSSION

In the present study, we found that the delivery of a costimulatory signal to resting human lymphocytes via an anti-FBP/anti-CD28 bi-mAb, together with the primary stimulation provided by the anti-FBP/anti-CD3 bi-mAb OC/TR, supported activation and proliferation of PBMCs in the presence of FBP^+ tumor cells, without exogenous growth factors such as IL-2. The resulting effector cells showed a potent ability to inhibit the growth of tumor cells *in vitro*, mainly by releasing soluble factors, which also exerted a bystander effect.

Our data demonstrate that the triggering of a lytic response depends strictly on cross-linking of the TCR/CD3 complexes by the anti-CD3-containing bi-mAb and cannot be further enhanced by cross-linking of the CD28 homodimers. Similarly, Kroesen *et al.* (24) found that CD28 costimulation does not increase anti-CD3/anti-TAA-mediated cytotoxicity of resting peripheral blood lymphocytes against the relevant target. The redirected lytic activity of resting mononuclear cells is confined to the CD8-enriched population, in agreement with previous reports (23, 34).

Engagement of the CD28 homodimer via an anti-CD28 mAb or FBP/CD28 bi-mAb greatly enhances the ability of OC/TR-retargeted resting PBMCs to block the growth of FBP^+ target cells *in vitro*, with the bimAb being the more potent. Both the $CD8^+$ and $CD4^+$ T-cell populations appear to be involved in the inhibition of tumor cells *in vitro*. The bystander effect, as well as the ability of supernatants to

Table 3 *Analysis of activity of PBMC supernatants after 3 days of culture*[a]

mAb[c]	% tumor growth inhibition of:[b] OVCAR3	A431	TNF-α (pg/ml)	IFN-γ (pg/ml)
None	0.25 (0–1)	7.2 (0–18)	4 (0–12)	6 (0–22)
OC/TR	12.5 (5–21)	13.2 (0–29)	321 (95–670)	890 (745–967)
OC/TR + αCD28	21.0 (13–33)	33.5 (17–53)	905 (166–1559)	4770 (4195–5096)
OC/TR + FBP/CD28	28.7 (21–40)	57.5 (31–79)	1985 (794–3572)	>>5000
αCD28	10.7 (4–16)	8.7 (1–14)	166 (36–174)	347 (7–518)
FBP/CD28	13.2 (3–23)	12.5 (6–18)	41 (10–61)	105 (0–321)

[a] PBMCs were cocultured for 3 days with FBP^+ target cells (E:T ratio, 20:1) and the various mAb combinations. On day 3, supernatants were collected, filtered, and analyzed. Data represent the mean of four to six different donors. The range is given in brackets.
[b] Evaluated in an MTT assay. Supernatants were diluted 2-fold.
[c] mAb used for stimulation. All mAbs were in $F(ab')_2$ form.

inhibit the growth of FBP^+ and FBP^- target cells, provide evidence that in the case of CD3/CD28 costimulation of resting PBMCs, the *in vitro* antitumor activity is mainly mediated by soluble factors released by lymphocytes upon triggering of their TCR/CD3 complexes, as described previously for bi-mAb-retargeted activity of activated T cells (35, 36). Indeed, IFN-γ and TNF-α, both reported to be highly cytostatic (11, 35), were detected at high titers in the culture medium derived from OC/TR plus FBP/CD28 bi-mAb-stimulated PBMCs. The release of TNF-α and IFN-γ from activated T cells has been reported to follow different kinetics; TNF-α is detectable in the medium as early as 6 h after stimulation, because it does not require new protein synthesis, whereas IFN-γ secretion is delayed and becomes detectable about 48 h after activation. Findings in our system were consistent with the pattern of secretion described (37): TNF-α was present in the supernatants after 24 h of stimulation, and the levels were comparable to those detected on day 3, whereas IFN-γ was detectable only on day 2 (data not shown).

The phenomenon of the bystander effect is particularly important in the context of therapeutic application, because the triggering of the TCR/CD3 complexes would occur at the tumor site via the anti-CD3/anti-TAA bi-mAb (OC/TR in our system); this would allow the local release by the effector cells of inhibitory cytokines (or lytic proteins) able to inhibit the growth of virtually all cells within the tumor, including those sterically inaccessible to targeted PBMC or those that have lost antigen expression. The local release of cytokines could trigger an inflammatory reaction, recruiting other components of the immune system to the tumor site. The addition of an anti-CD28/anti-TAA bi-mAb (our FBP/CD28 bimAb) could further enhance cytokine release and the effectiveness of the treatment. Simultaneous ligation of CD28 and TCR/CD3 has been shown to augment dramatically the production of a variety of cytokines, including IL-2, TNF-α, IFN-γ, and granulocyte-macrophage colony stimulating factor, by preventing their mRNA degradation and increasing the transcription rates of the corresponding genes (37, 38).

The enhanced tumor growth-inhibitory effects and the enhanced cytokine release observed in the presence of the CD28 costimulus correlated with an increase of the lymphocyte activation state. Our data on CD69 expression, *i.e.*, increase at 6 h and persistence up to day 3, indicate that the kinetics of expression of this early activation marker (39) after CD3/CD28 stimulation are different from those following mitogenic or antigenic stimulation, where CD69 is reportedly maximum after 6–8 h and returns to baseline levels after 36–48 h (40).

The α subunit of the IL-2 receptor (CD25) is undetectable on resting T cells but is efficiently induced upon T-cell activation by different stimuli, including anti-CD3 mAb (41). OC/TR alone elicited a detectable up-modulation of CD25 in T cells after 3 days of stimulation in the presence of FBP^+ target cells, which was slightly increased in the presence of anti-CD28 mAb but dramatically increased with the combination of OC/TR and FBP/CD28 bi-mAb. Liu and Janeway (42) and more recently Levine *et al.* (43) reported that CD3/CD28 costimulation is more efficient when both the primary and the costimulatory signals are provided by the same antigen-presenting cell ("*cis*" stimulation) rather than distinct antigen-presenting cells ("*trans*" stimulation). Our data are consistent with the concept of *cis* stimulation of T cells obtained with the two bi-mAb together, whereas *trans* stimulation through the combination of OC/TR plus soluble anti-CD28 mAb was less active.

Ligation of CD28 by the bivalent anti-CD28 mAb, but not by the FBP/CD28 bi-mAb, independent of the presence of OC/TR, always resulted in a 100% down-regulation of CD28 from the surface of T cells, which returned to baseline levels only 7 days after stimulation. Despite the absence of CD28 from their surface, PBMCs cocultured for 3 days with FBP^+ target cells, together with OC/TR and anti-CD28 mAb, have detectable antitumor activities, consistent with the observations of Azuma *et al.* (44) that CD28 costimulation, although necessary for the induction of a state of activation, is not required for T cell effector functions.

Besides the enhanced *in vitro* antitumor activity and the enhanced lymphocyte activation state induced by the CD28 costimulus, other observations indicate the possibility of an *in vivo* application of this costimulation setting.

(*a*) The bi-mAb OC/TR was detectable on the surface of PBMCs after 3 days of coincubation with FBP^+ target cells and different mAb combinations and was still able to redirect their activities against FBP^+ target cells in both cytotoxicity and MTT assays. These data conflict with previous observations that incubation of T-cell clones with OC/TR and FBP^+ target cells results in a rapid loss of bi-mAb redirected cytotoxicity against FBP^+ target cells, even if the bi-mAb is still present on the surface of T cells (45). The inability of the TCR/CD3 complexes bound to the bi-mAb to transduce activation signals following cross-linking with FBP^+ cells was ascribed to their clustering. The different T-cell preparations (PBMCs instead of T-cell clones), as well as the different experimental conditions and absence of exogenous IL-2 in our system, may account for the observed difference. The antitumor activities of OC/TR-activated PBMCs are only slightly increased by the addition of new OC/TR; therefore, we can hypothesize that in our conditions, the TCR/CD3 complexes do not lose or lose only partially their ability to transduce activation signals when cross-linked to target cells via the residual bi-mAb OC/TR.

(*b*) After coculture with FBP^+ cells, only PBMCs incubated with the two bi-mAbs showed a significant proliferation over a 10-day period, a phenomenon which probably determined a shift in the E:T ratio and thus a higher antitumor activity. No exogenous IL-2 was added to the culture medium; therefore, cell proliferation was entirely dependent on the release of growth factors by lymphocytes. The signal delivered by soluble anti-CD28 mAb $F(ab')_2$ enabled OC/TR-coated PBMCs to gain an activation status (as judged by CD25 up-regulation) and to release cytokines that exerted an antiproliferative effect on tumor cells; yet it was not strong enough to allow a self-sustained proliferation. Only a cross-linked anti-CD28 mAb (our FBP/CD28 bi-mAb) provided PBMCs with a prolonged stimulus, which resulted in IL-2 secretion and autocrine stimulation. Similarly, Levine *et al.* (43) and Kroesen *et al.* (24) found that no IL-2 was necessary to support the proliferation of CD3-stimulated T cells after costimulation via cross-linked CD28 molecules.

To date, in the single report describing the combined use of anti-CD3/anti-TAA and anti-CD28/anti-TAA bi-mAbs for the treatment of established human tumors in an *in vivo* model (25), the effector cells were previously activated *in vitro* for 3 days with the anti-CD3/anti-TAA bi-mAb in the presence of tumor target cells. Because our *in vitro* data indicated that cross-linking between tumor and effector cells for 24 h is sufficient to achieve T-cell activation and development of antitumor activities, it is reasonable to hypothesize that a short-term preactivation of PBMCs will be effective in the treatment of the tumor in an *in vivo* setting. Thus, experiments are under way in which a short-term (24-h) *in vitro* stimulation of resting PBMCs in the presence of fixed FBP^+ tumor cells and the two bi-mAbs (OC/TR and FBP/CD28) together is performed prior to injection into tumor-bearing nude mice.

Even if a preactivation of effector cells is probably unavoidable, it is reasonable to conceive that the dramatic reduction in the activation period and the self-sustained proliferation achieved via CD3/CD28 costimulation will yield a significant improvement in bi-mAb-based immunotherapy. We suggest that an *ex vivo* coating of resting PBMCs

and short *in vitro* stimulation could be of therapeutic utility for the local treatment of minimal residual disease. It will be of significant interest to evaluate this approach in patients and relate corresponding clinical data to predictions from *in vitro* and *in vivo* assays.

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